# Tall cell Papillary Thyroid Carcinoma: new diagnostic criteria and mutations in BRAF and TERT

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Abstract: OBJECTIVE: The tall cell variant of papillary thyroid carcinoma (PTC) has an unfavorable prognosis. The diagnostic criteria remain inconsistent and the role of a minor tall cell (TC) component is unclear. Molecular diagnostic markers are not available; however, there are two potential candidates: BRAF V600E and TERT promoter mutations. METHODS: Using a novel approach, we enriched a collective with PTC harboring an adverse outcome, overcoming the limited statistical power of most studies. This enabled us to review 125 PTC patients, 57 of them with an adverse outcome. The proportion of TC that constitute a poor prognosis was assessed. All tumors underwent sequencing for TERT promoter and BRAF V600E mutational status and were stained with an antibody detecting the BRAF V600E mutation. RESULTS: A 10% cut off for TC was significantly associated with advanced tumor stage and lymph node metastasis. Multivariate analysis showed that tall cells above 10% were the only significant factor for overall, tumor-specific and relapse-free survival. 7% of cases had a TERT promoter mutation while 61% demonstrated a BRAF mutation. The presence of TC was significantly associated with TERT promoter and BRAF mutations. TERT predicted highly significant tumor relapse ($p<0.001$). CONCLUSION: PTC comprising of at least 10% TC are associated with an adverse clinical outcome and should be reported accordingly. BRAF did not influence patient outcome. Nevertheless, a positive status should encourage the search for tall cells. TERT promoter mutations are a strong predictor of tumor relapse but their role as a surrogate marker for TC is limited.

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# Tall cell Papillary Thyroid Carcinoma: new diagnostic criteria and mutations in *BRAF* and *TERT*

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Running title: Tall cells, *TERT* and *BRAF* in PTC

**Disclosure/Conflict of Interest**

David Capper declares shared inventorship of VE1 antibody. A patent for diagnostic use of VE1 has been applied for. Under a licensing agreement between Ventana Medical Systems Inc., Tucson, Arizona, and the German Cancer Research Center, David Capper is entitled to a share of royalties received by the German Cancer Research Center on the sales of VE1 antibody. The terms of this arrangement are being managed by the German Cancer Research Center in accordance with its conflict of interest policies.

**Abstract**

The tall cell variant of papillary thyroid carcinoma (PTC) has an unfavorable prognosis. The diagnostic criteria remain inconsistent and the role of a minor tall cell (TC) component is unclear. Molecular diagnostic markers are not available; however, there are two potential candidates: *BRAF V600E* and *TERT* promoter mutations. Using a novel approach, we enriched a collective with PTC harboring an adverse outcome, overcoming the limited statistical power of most studies. This enabled us to review 125 PTC patients, 57 of them with an adverse outcome. The proportion of TC that constitute a poor prognosis was assessed. All tumors underwent sequencing for *TERT* promoter and *BRAF V600E* mutational status and were stained with an antibody detecting the *BRAF V600E* mutation. A 10% cut off for TC was significantly associated with advanced tumor stage and lymph node metastasis. Multivariate analysis showed that tall cells above 10% were the only significant factor for overall, tumor-specific and relapse-free survival. 7% of cases had a *TERT* promoter mutation while 61% demonstrated a *BRAF* mutation. The presence of TC was significantly associated with *TERT* promoter and *BRAF* mutations. *TERT* predicted highly significant tumor relapse ($p<0.001$). PTC comprising of at least 10% TC are associated with an adverse clinical outcome and should be reported accordingly. *BRAF* did not influence patient outcome. Nevertheless, a positive status should encourage the search for tall cells. *TERT* promoter mutations are a strong predictor of tumor relapse but their role as a surrogate marker for TC is limited.

## Introduction

Thyroid carcinoma is classified into five different morphological groups: papillary, follicular, medullary, poorly differentiated and anaplastic/undifferentiated(DeLellis, et al. 2004). These endocrine cancers account for approximately 1% of all malignancies(Schlumberger 1998). Papillary thyroid carcinomas (PTC) are among the most curable cancers and total thyroidectomy followed by adjuvant radioiodine administration cures almost all patients. Patient prognosis depends upon age, gender, tumor size or extrathyroidal extension, the latter two of which are reflected in the UICC TNM staging system(Sobin S. 2009). On a morphologic basis, PTC are subdivided into different subtypes as recognized by the WHO-classification(DeLellis et al. 2004). Nevertheless, this sub-classification has little clinical significance since the different subtypes do not add independent prognostic value. As a result the vast majority of PTC is not sub-classified routinely. A grading system of PTC has been proposed that included nuclear atypia, tumor necrosis and vascular invasion as signs for poorly differentiated PTC(Akslen and LiVolsi 2000). However most of these tumors would nowadays probably be classified as poorly differentiated thyroid carcinomas(Volante, et al. 2007). Different staging systems for PTC have been introduced. Unfortunately, they are very complex and as a consequence, they are not used on a daily basis in most institutions. In fact, it is very difficult to predict which patients will suffer from recurrent disease or death from PTC and which will be cured by the combination of surgery and radioiodine treatment(Nikiforov 2012).

Among the different PTC subtypes, the tall cell variant (TCV) is known to have a more aggressive clinical behavior(LiVolsi 2010). However, the morphological criteria for the diagnosis of this entity is not clear defined by the WHO (“The TCV is composed predominantly of cells whose heights are at least three times their widths.”) and especially the percentage of tall cells (TC) needed in a given tumor is defined variably in the literature(DeLellis et al. 2004; Ostrowski and Merino 1996).

The oncogenic *BRAF V600E* mutation occurs in about 40-45% of PTC(Nikiforov 2012). It activates the mitogen-activated protein kinase signaling pathway (MAPK) in human cancer and has been shown to correlate with aggressive features in PTC including extrathyroidal extension, lymph node metastasis and the TCV(Kim, et al. 2012; Koperek, et al. 2012; Li, et al. 2012; Xing, et al. 2013).

An increasing number of studies including meta-analyses were able to demonstrate an association between *BRAF* status and aggressive tumor behavior(Elisei, et al. 2012; Kim et al. 2012; Li et al. 2012; Xing et al. 2013). Other studies, however, could not confirm this data, resulting in uncertainty of the value of *BRAF* in PTC (Barbaro, et al. 2014; Cheng, et al. 2011; Eloy, et al. 2011; Ito, et al. 2009; Koperek et al. 2012; Sancisi, et al. 2012). One reason for this could be the fact that an adverse patient outcome with multiple tumor relapses and eventually death due to PTC is a rare event. As a matter of fact, only about 5% of PTC will have an adverse outcome in consecutive clinicopathological series, leading to very small groups of high-risk tumors with limited statistical power in different studies(Handkiewicz-Junak, et al. 2010).

Reactivation of the telomerase reverse transcriptase (*TERT*) gene which encodes for the catalytic subunit of telomerase is implicated in tumorigenesis and cell immortalization. The two promoter mutations C228T and C250T were recently reported in human melanomas and other human cancer types including thyroid(Huang, et al. 2013; Liu, et al. 2013a; Vinagre, et al. 2013). They are a novel mechanism in tumor biology, generating de novo consensus binding motifs for ETS (E-twenty-six) transcription factors, increasing the activity of the *TERT* promoter with potential clinical applications and prognostic value(Huang et al. 2013; Landa, et al. 2013; Liu, et al. 2013b).

The purpose of this study was to analyze the prevalence, histomorphology and follow-up data of a cohort of PTC patients enriched for patients with known adverse clinical outcome (ACO) based on a population of 1.3 million inhabitants over a 16 year period and to determine the threshold of TC within a given PTC that could impact patient outcome. Further, we sequenced *BRAF* and *TERT* promoter mutations in all tumors and evaluated whether they may serve as surrogate markers for TCV and an ACO.

## Material and Methods

Since radioiodine treatment is the standard therapy for patients suffering from recurrent thyroid cancer, virtually all such patients are seen in the department of nuclear medicine. Three such departments (University Hospital Zürich, City Hospital Triemli, and Cantonal Hospital Winterthur) serve the 1.3 million inhabitants in the canton of Zürich(Statistics 2011). Fifty-seven patients were

identified in these departments who had thyroid surgery between 1990 and 2006 and had an ACO. ACO was defined as more than one tumor recurrence or tumor related death. Sixty-eight age-, stage- and gender-matched patients (without recurrence) treated for PTC at the University Hospital Zürich were used as the control group (CG; table 1). Tumors with a size of less than 1 cm were excluded from this study since these microcarcinomas show a very indolent clinical course(Baloch and LiVolsi 2006) and the significance of TC in this setting is beyond the scope of this study. This approach significantly enhanced the number of patients with an ACO. Twenty one of the patients had lymph node metastases whereas 104 patients were node negative.

Archival pathology specimens of the thyroid tumors were reevaluated according to the WHO classification by three pathologists who were blinded to the clinicopathological data (M.D., A.S., A.P.) and a consensus diagnosis was rendered(DeLellis et al. 2004). The tumor stage was reclassified based on the 7th TNM classification(Sobin S. 2009). TC were defined as cells being more than three times as high as wide, often with an eosinophilic cytoplasm(Nikiforov 2012). A few examples and pitfalls are shown in figure 1. Although the so-called “tram-track” pattern, especially, at low power is a very helpful feature, not all areas showing this growth pattern are TC. Also areas exhibiting a classic PTC growth pattern can be comprised of true TC, thus making it mandatory for the pathologist to examine all tumors at high magnification. Nuclear stratification, intranuclear inclusions as well as characteristic coffee bean infoldings of the nuclear membrane were frequently seen(Nikiforov 2012).

The percentage of TC was recorded semiquantitatively in each tumor in 10% increment steps, ranging from 0-100%, the lowest recorded amount of TC in a given tumor was therefore 10%. All available slides were assessed in each tumor (6.1±4.5 slides per case in the CG and 5.0±4.5 slides in the ACO group). Tumors which had anaplastic or poorly differentiated areas, as defined by the Turin criteria were excluded from the study(Volante et al. 2007), and were reported elsewhere (Dettmer, et al. 2011; Dettmer, et al. 2012).

DNA was extracted using the DNeasy Blood and Tissue kit and all samples underwent *BRAF* pyrosequencing mutation analysis on the PyroMark Q24 System according to the manufacturer’s instructions (QIAGEN, Hilden, Germany). The primers had the following sequence: forward: 5’-TGAAGACCTCACAGTAAAAATAGG-3’; reverse: 5’-TCCAGACAACTGTTCAAACTGAT-3’;

sequencing primer: 5'-TGATTTTGGTCTAGCTACA-3'. An allelic fraction of ≥10% of mutant alleles was defined as positive for the mutation.

A tissue microarray (TMA) has been constructed and stained with the *BRAF V600E* mutation specific antibody as previously described (Boos, et al. 2013; Koperek et al. 2012). A tumor was considered positive when a distinct, homogenous immunoreaction was seen in the cytoplasm of all cancer cells. Immunohistochemistry and molecular analysis were performed on the same tissue block.

*TERT* promoter mutations were assessed by PCR followed by Sanger Sequencing as described (Liu et al. 2013a).

The sample distribution was analyzed using the Kolmogorov-Smirnov test. Chi-square, Kendall's tau_b, Kruskal-Wallis, Kaplan-Meier and COX regression were performed using SPSS statistical software version 21.0 (SPSS, Chicago, IL). A *P* value of <0.05 was considered statistically significant. The study was approved by the Cantonal Research Ethics Board Zürich (STV 28-2006).

**Results**

Fifty-seven PTC patients with ACO and sixty-eight PTC patients in the CG were analyzed. The characteristics of the study population are summarized in table 1 and 2. The mean±st.dev. for the different follow up times in months were: OS (overall survival): 81.97±48.42; TSS (tumor specific survival): 81.97±48.42; RFS (relapse free survival): 51.78±50.97; CG 81.88±48.5; ACO 81.5±49.5. There were 68 classic PTC, 47 follicular variants of PTC (FVPTC) and 10 PTC of a special subtype (3 diffuse sclerosing, 2 columnar cell, 2 solid, 2 warthin-like and one oncocytic variant). Seventy-seven PTC did not display any detectable TCs, 27 PTC showed more than 10% but less than 50% TC and 21 tumors had ≥50% of tumor composed of TC. The distribution of TC and important clinical endpoints OS, TSS and RFS are summarized in table 2 and table suppl.1.

The tumors comprising of 10- 50% TC showed the presence of TC predominantly at the invasive front, whereas tumors harboring ≥50% TC showed presence of tall cells in the center of the tumor as well.

**Tall cells:** The presence of TC showed significant correlation with patient survival, relapse and tumor stage. Clinicopathological data for the tumors were also correlated with the different

thresholds for TC presence in the tumors and ROC curves were calculated to assess the best cut-off (fig suppl.1; table suppl.2). TC >10% correlated with lymph node metastasis ($p<0.001$) but not with organ metastases ($p<0.223$). TC ≥50% did not correlate with lymph node metastasis ($p<0.141$) nor with organ metastasis ($p<0.601$).

Kaplan-Meier survival analysis showed significant reduction in OS, TSS and RFS for tumors having more than 10% TC (each: Log-Rank $p<0.001$). To further determine what proportion of ≥10% TC impacts patient prognosis, we subcategorised this group into two groups, namely 10-50% and ≥ 50% TC area. There were no significant differences between the two groups with regards to OS, TSS and RFS, but in the group with 10% to 50% TC there was a tendency for less aggressive behaviour as compared to the group with ≥ 50% TC area (figure 2).

Multivariate Cox regression analysis was performed using patient age, gender, tumor stage and TC tumor area (<10% vs. ≥10%) for OS, RFS and TSS. For RFS, TC ≥ 10% was the only parameter that was significant ($p<0.0001$, Exp(B)=3.6). For OS, the factors that were significant were age ($p<0.010$, Exp(B)=6.3) and TC ≥ 10% ($p<0.001$, Exp(B)=12.9). The analysis could not be performed for TSS, since no patient in the TC<10% group died. However when the analysis for TSS was performed using the threshold of TC ≥ 50%, it was significant ($p<0.008$, Exp(B)=9.3). The Kruskal-Wallis test revealed a significant association between the number of relapses and the percentage of TC found in a given tumor ($p<0.001$; table suppl.3).

***BRAF*:** Pyrosequencing analysis detected *BRAF V600E* in 72 (61%) PTC cases whereas 46 tumors (39%) were negative. All the mutations were *V600E*, other *BRAF* mutations were not detected. The sensitivity was higher as compared to the mutation specific antibody which detected the mutation in 63 (50.4%) cases and which was negative in the other 62 (49.6%) patients. The allelic fraction of *BRAF* (29.4±13.5, range 10 to 46) mutation did not correlate with patient outcome. Pyrosequencing was more sensitive in nine discrepant cases and detected the *BRAF* mutation while the antibody was negative. Two of these cases had a subsequent lymph node metastasis, which was then positive for *BRAF* by the antibody. Only two tumors showing a positive IHC for *V600E* were negative by Pyrosequencing. In summary, there was an excellent highly significant correlation between both detection methods ($p<0.001$, $r=0.600$, table suppl.2).

*BRAF* status was not associated with patient outcome in the Kaplan Meier analysis or the Cox regression multivariate analysis (figure 3). There were no associations of *BRAF* mutations with lymph node or organ metastases.

**Tall cells and *BRAF*:** Sixteen out of 21 patients (76%) had the *BRAF* mutation when applying the 50% cutoff for the TCV. This went up to 79% (38 out of 48 patients) when applying the 10% cutoff to be required to allow the TCV diagnosis. The amount of TC within a given tumor was significantly associated with the *BRAF* status ($p<0.002$, table 3 and table suppl.1).

The allelic fraction as determined by pyrosequencing was influenced by the amount of TC within a tumor ($p<0.034$, $r=0.156$; Kendall's tau_b). There was no significant association between *BRAF* status and an ACO on univariate or multivariate analysis when we analyzed only the TC tumors, neither for the 10% nor for the 50% cut-off (table 1, table suppl.2, table suppl.3).

**Tall cells, *BRAF* and *TERT*:** *TERT* promoter mutations were detected in total 8 tumors (7.3%). The C250T mutation was found in three tumors, the C228T mutation in five. Six patients with *TERT* promoter mutations were also *BRAF* mutated while two were not ($p<0.49$). TC morphology correlated significantly with this mutation ($p<0.0001$, $r=0.308$) - six of these tumors had a TC morphology in ≥ 50% whereas one had 30% TC and one had none. All eight patients with the *TERT* promoter mutation had a tumor relapse ($p<0.001$). Tumors that were *TERT* promoter mutated and *BRAF* mutated behaved significantly worse than *BRAF* mutated tumors only (Log-Rank $p<0.019$; table 1 and table suppl.2).

*TERT* promoter mutations together with pT stage remained an independent predictor of tumor relapse in a multivariate analysis (*TERT* promoter mutation: $p<0.005$; Exp(b)=3.18; pT stage: $p<0.019$; Exp(b)=1.58). However, when TC were included in the analysis, they remained the single independent predictor of RFS ($p<0.0001$; Exp(b)=3.04) while the other two factors dropped out (table suppl.3).

The numbers of some parameters do not add up to the total number of patients included in the study due to dropouts in DNA quality and consecutive failure of mutational testing or due to dropout of cases on the tissue microarray.

228

229 **Discussion**

230 In this study, we analyzed a large cohort of PTC and TCV with an ACO, delineated the
231 proportion of TC that is needed to determine patient outcome and assessed the role of *BRAF* and *TERT*
232 promoter mutations in this setting. In our hands a 10% cut-off of TC defines a detrimental prognosis.
233 *BRAF* mutation status does not correlate with adverse outcome, but is more frequent in TCV than in
234 classical PTC. *TERT* mutations are rare, but associated with a very aggressive course.

235 Previous clinico-pathological studies on TCV PTC analyzed small groups of tumors with
236 ACO thereby limiting their statistical power(LiVolsi 2010; Ostrowski and Merino 1996). In our study
237 this number of patients with ACO was increased to a relatively large sample size via identification of
238 patients in the departments of nuclear medicine. This approach increased the statistical power in
239 determining a cut-off of TC leading to poor prognosis. This patient selection is on the other hand also
240 a limitation of the study since it does not reflect the normal patient population. We can therefore not
241 exclude that we overestimated the effect of TC in tumors with an ACO and that TC are much more
242 frequently encountered in a consecutive series of patients where they do not account for an ACO.
243 However, in our age, stage and gender matched cohort, this was not the case.

244 The TCV of PTC is a distinct entity of thyroid tumors that is associated with an unfavorable
245 patient outcome. The threshold of proportion of TC in a given carcinoma to allow for the diagnosis of
246 TC-variant is poorly defined and differs in the literature from no information at all to 10% up to
247 75%(Akslen and LiVolsi 2000; DeLellis et al. 2004; LiVolsi 2010; Ostrowski and Merino 1996;
248 Sobrinho-Simoes, et al. 1989). Our results confirm that PTC comprising more than 50% TC have an
249 ACO. While this result was expected, it settles the finding in a robust study population. Survival
250 analysis showed that there was a clear difference between the TC and non-TC PTC using this 50%
251 cut-off. However, the results were more interesting when a cut-off at 10% was introduced, since the
252 influence of a minor TC component is unclear and controversial (LiVolsi 2010; Nikiforov 2012). To
253 exclude the possibility, that PTC comprising of TC ≥50% group skewed the analysis and that TC
254 component 10-50% did not severely affect patient outcome, we separated the tumors into three groups,
255 <10% TC, 10-50% TC, and ≥50% TC. The Kaplan-Meier analysis revealed that presence of at least

10% of TC in a PTC significantly impacted the patient outcome for OS, TSS and RFS. Moreover, TC≥10% is the only significant factor in a multivariate analysis, which included age, gender and tumor stage, for ACO.

It has already been proposed by Sobrinho and others that it is important for pathologists to report the number of TC seen in a given tumor, few as they may be(LiVolsi 2010; Sobrinho-Simoes et al. 1989). Two studies suggested that a minor TC component may determine patient outcome but they were not able to confirm this in a univariate respectively multivariate analysis(Beninato, et al. 2013; Ganly, et al. 2013). The first study did a review on cases that had been diagnosed as TCV or PTC with a minor TC component(Beninato et al. 2013). With such an approach, we would have missed most of the TCV in our study. In fact, only three PTC consisting exclusively of TC were diagnosed as TCV in the original pathology report. All remaining PTC were originally diagnosed as either classical PTC or FVPTC, even when a threshold of 50% was applied. These underdiagnoses could be due to their rarity and lack of awareness on the part of the surgical pathologists on the one hand or due to the ambiguous definition of the condition on the other. The second study defined TC as cells with their height twice their width and therefore included tumors as TCV, which would not be included as TCV in the present work. Our results indicate that the strict application of the 2004 WHO definition of TC with cells being thrice their width stratifies patients better(DeLellis et al. 2004). This study also had only few patients with adverse events and due to those reasons was probably therefore not significant in the multivariate analysis(Ganly et al. 2013).

From our study, it is evident that presence of TC component of 10-50% significantly and independently affects patient prognosis and that there is no statistical difference between the group with a 10-50% TC and ≥ 50% TC component. The PTC with 10% to 50% of TC would be regarded as conventional PTC or as PTC with TC features by most authors(Ghossein and Livolsi 2008; LiVolsi 2010). At this point, it is important to specify that we examined approximately 6 tumor blocks per patient and that examining only one block per tumor would have lowered the rate of detection of TCV by 11%, using the 10% cut-off. To our knowledge, no image analysis tool is available that would be able to identify TC within a PTC and quantify their amount in a given tumor.

The Kaplan-Meier survival analysis in our study suggests that there might be a difference between the tumors harboring a 10-50% TC versus a ≥ 50% TC component, with the former showing a tendency for less aggressive behavior. We propose two potential explanations for this finding. Firstly, there could be a limitation in morphological detection of TC areas in the sub 10% range which can lead to these tumors being categorized in the 10% to 50% TC group, thereby accounting for the intermediate results in the Kaplan-Meier analysis. This also implies that the greater the TC component that was identified, the higher the possibility that this tumor was truly a TC variant of PTC. The second explanation for this finding is that there could be a gradual increase of TC leading to a gradually increased aggressiveness. Another argument for the second explanation is the comparison of TCV to other solid carcinomas. Different subclones in tumors play an important role in the formation of tumor progression and metastasis (Yachida, et al. 2010). Such more aggressive subclones could gradually replace the less aggressive clones. A potential driver mutation for TCV PTC might be the *BRAF V600E* mutation. Indeed, the present work confirms the fact that *BRAF* mutations are more frequently observed in TCV as compared to PTC(Nikiforova, et al. 2003). Further, it was demonstrated that PTC showing *BRAF* mutations have a higher frequency of extrathyroidal extension and nodal metastases than those that are negative for this mutation(Nikiforova et al. 2003).

While our study was not designed to analyze genetic intratumoral heterogeneity, we saw 11 of 125 tumors with different *BRAF* mutation results in 2 regions and the immunohistochemical *BRAF V600E* detection was heterogeneous in two cases while the allelic fraction by pyrosequencing was above 30% in both cases. These findings support the fact that *BRAF* tumor heterogeneity occurs in PTC(Guerra, et al. 2012b) but its prognostic role is unclear, since the allelic fraction did not correlate with patient outcome which is in contrast to previous findings(Guerra, et al. 2012a). In fact, analyzing the whole group of PTC we did not detect any significant association between the *BRAF* status of the tumors and patient outcome, neither when we analyzed all tumors, nor when we analyzed only the TCV. These findings are supported by other groups which were also not able to find such a relationship(Barbaro et al. 2014; Eloy et al. 2011; Ito et al. 2009; Koperek et al. 2012; Sancisi et al. 2012). Epidemiologically, it is not surprising that the *BRAF* mutation does not predict ACO, since *BRAF* mutations can be found in about 40-50% of PTC, whereas less than 5% of these tumors have an

aggressive clinical course. Therefore, we believe that additional factors have to be present that account for the ACO in patients harboring this mutation.

One of these potential additional factors could be *TERT* promoter mutations. They were recently discovered to be a novel mechanism of telomerase activation, which is known to be centrally involved in the tumorigenesis of various human cancer types including thyroid(Vinagre et al. 2013). From a diagnostic standpoint of view, it is worthwhile to mention that *TERT* promoter mutations were not detected in medullary thyroid carcinomas, nor in benign lesions or normal thyroid(Vinagre et al. 2013). More important, recent studies including TCGA data reported *TERT* promoter mutations to play a role in aggressive thyroid cancers and in tumor progression from well differentiated to poorly and anaplastic thyroid carcinomas. The various studies found a frequency in PTC ranging from 8-25% and to being associated with an unfavorable prognosis(Cancer Genome Atlas Research 2014; Liu et al. 2013a; Liu et al. 2013b; Melo, et al. 2014; Shimamura, et al. 2014; Vinagre et al. 2013). They occur significantly more often in TCV (30%) compared to conventional PTC or FVPTC (10%), and in poorly differentiated thyroid carcinomas (21-37%) compared to the differentiated subtypes (PTC or FTC: 12%) and have a high incidence in anaplastic thyroid carcinomas (13-46%)(Liu et al. 2013b; Vinagre et al. 2013). Although *TERT* promoter mutations significantly contribute to an ACO in PTC patients, many PTCs with an ACO including most TCV were *TERT* wild type. Thus, other molecular players being responsible for TC morphology and an ACO are yet to be discovered.

**Conclusions**

We show that presence of as few as 10% of TC within a PTC significantly affects patient prognosis. Therefore, these tumors should be classified accordingly and reported by the pathologist so the treating clinicians are aware that they are dealing with an aggressive neoplasm. The *BRAF* mutation did not affect patient outcome. Nevertheless, a mutation analysis for *BRAF* might be helpful in clinical management since a positive *BRAF* status may trigger the search for TC in order to predict patient outcome and new therapeutics inhibiting the mutated protein are now clinically available. *TERT* promoter mutations are too rare to serve as a surrogate marker for TC. However, they are a

novel tool to predict molecularly an adverse outcome in a subset of patients and an implementation into daily molecular diagnostics is worthwhile.

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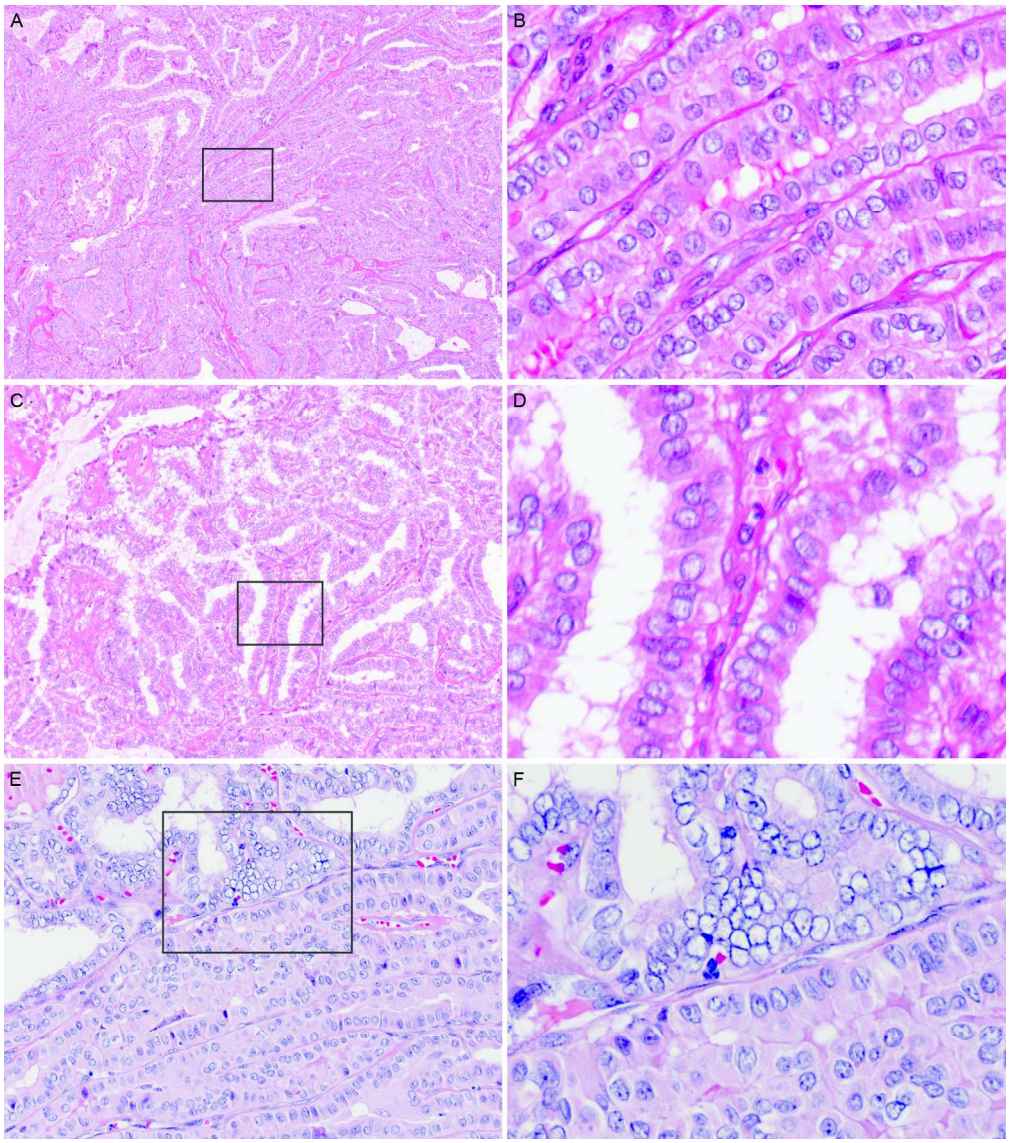


A: Papillary cancer; tall cell variant: tumor cells are at least thrice as height as their width and show an eosinophilic cytoplasm and elongated follicles (HE, 200x)
B: Same tumor as A: Papillary cancer; tall cell variant: three times as height as width cells arranged in a "tram track" pattern with eosinophilic cytoplasm (HE, 400x)
C: Papillary cancer; tall cell variant: papillary growth pattern with delicate branching of papillae (HE, 200x)
D: Same tumor as C: Papillary cancer; tall cell variant: three times as height as width cells, growing in a classic papillary fashion (HE, 400x)
E: Not all cells in these tumors are at least three times high as width. However, tumor cells growing in the "tram track" pattern should trigger a careful search for tall cells (HE, 200x)
F: Same tumor as E: Tumor cells are cut tangential to their base (upper). Only cells can be assessed where the basal membrane is seen (lower). These tumor cells are classic tall cells, often with nuclear grooves and elongated follicles (HE, 400x)
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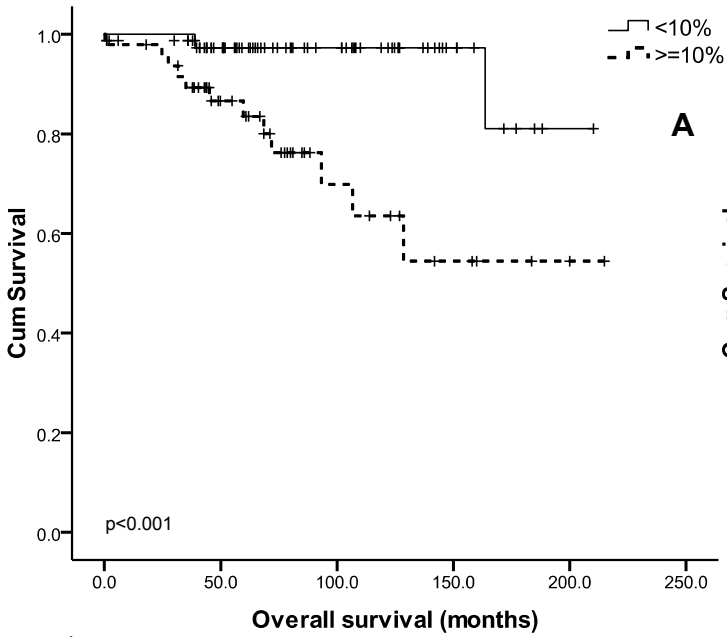
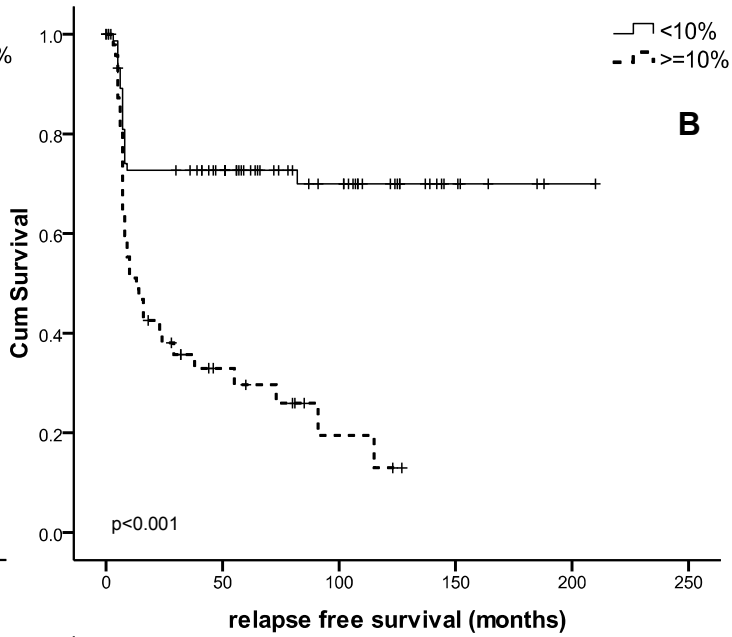
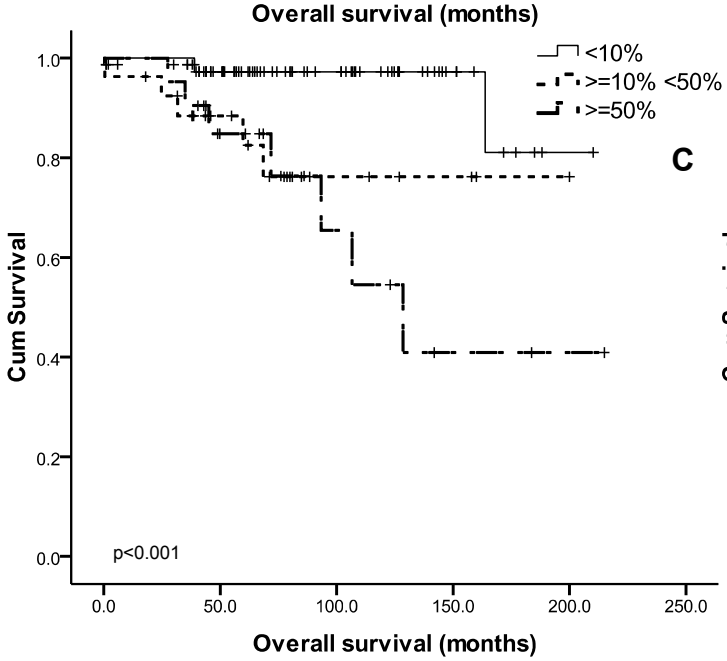
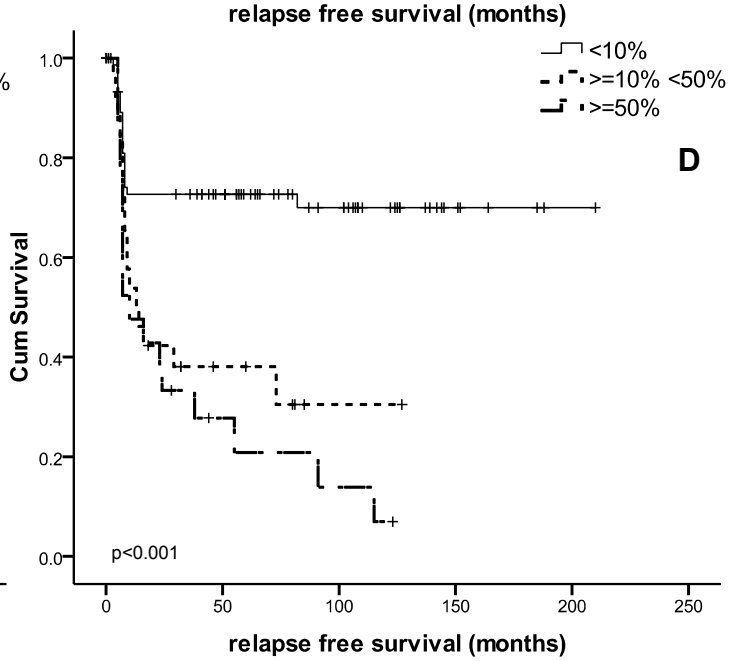

A
<10%
>=10%
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
p<0.001
0.0
50.0
100.0
150.0
200.0
250.0
Overall survival (months)


B
<10%
>=10%
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
p<0.001
0
50
100
150
200
250
relapse free survival (months)


C
<10%
>=10% <50%
>=50%
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
p<0.001
0.0
50.0
100.0
150.0
200.0
250.0
Overall survival (months)


D
<10%
>=10% <50%
>=50%
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
p<0.001
0
50
100
150
200
250
relapse free survival (months)

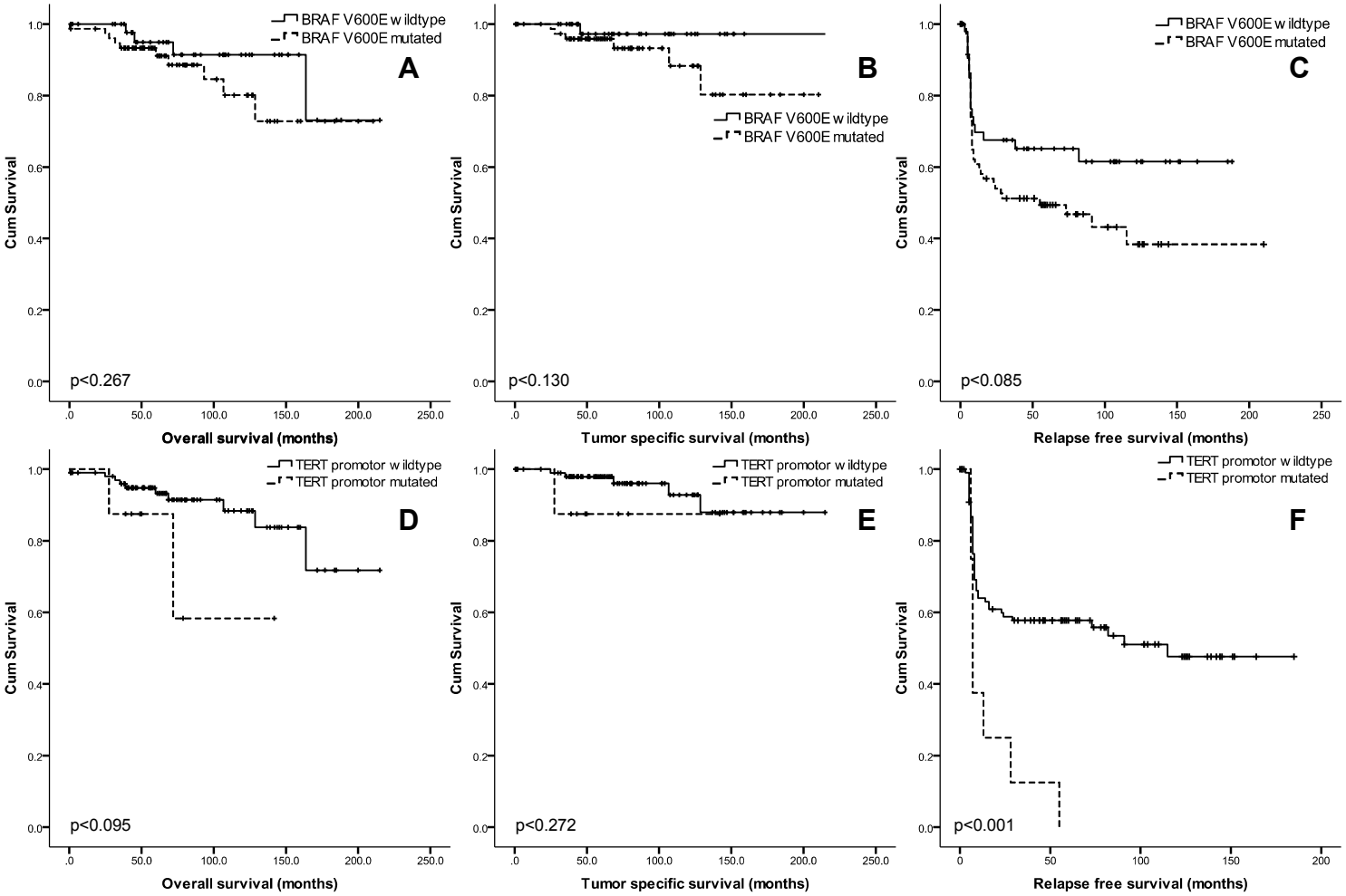

BRAF V600E wildtype
BRAF V600E mutated
A
Cum Survival
p<0.267
Overall survival (months)
B
p<0.130
Tumor specific survival (months)
C
p<0.085
Relapse free survival (months)
TERT promotor wildtype
TERT promotor mutated
D
p<0.095
E
p<0.272
F
p<0.001

Table 1: Clinicopathological data

| | | total (n) | ACO | CG | p-value |
|---|---|---|---|---|---|
| age | age <48 | 69 (55.2%) | 32 (25.6%) | 37 (29.6%) | 0.859 |
| | age ≥48 | 56 (44.8%) | 25 (20%) | 31 (24.8%) | |
| gender | male | 34 (27.2%) | 20 (16%) | 14 (11.2%) | 0.106 |
| | female | 91 (72.8%) | 37 (29.6%) | 54 (43.8%) | |
| pT | pT 1-2 | 55 (44%) | 18 (14.4%) | 37 (29.6%) | 0.012 |
| | pT 3-4 | 70 (56%) | 39 (31.2%) | 31 (24.8%) | |
| LN | no metastasis | 104 (83.2%) | 38 (30.4%) | 66 (52.8%) | 0.001 |
| | metastasis | 21 (16.8%) | 19 (15.2%) | 2 (1.6%) | |
| TC | TC <10% | 77 (61.6%) | 21 (16.8%) | 56 (44.8%) | 0.00001 |
| | TC ≥10% | 48 (38.4%) | 36 (28.8%) | 12 (9.6%) | |
| BRAF total* | wild type | 49 (39.2%) | 17 (13.6%) | 32 (25.6%) | 0.066 |
| | mutated | 76 (60.8%) | 40 (32.0%) | 36 (28.8%) | |
| TERT | wild type | 102 (92.7%) | 45 (40.9%) | 57 (51.8%) | 0.002 |
| | mutated | 8 (7.3%) | 8 (7.3%) | 0 (0%) | |

Chi Square test

Clinical and pathological characteritics between the adverse clinical outcome group (ACO) and the control group (CG)

* result of BRAF testing by pyrosequencing or by the VE1 antibody where no BRAF mutational testing by pyrosequencing was possible

Table 2: Clinicopathological data

| | | total (n) | TC <10% (n) | TC ≥10% (n) | p-value | TC <50% (n) | TC ≥50% (n) | p-value |
|---|---|---|---|---|---|---|---|---|
| age | age <48 | 69 (55.2%) | 44 (35.2%) | 25 (20%) | 0.586 | 62 (49.6%) | 7 (5.6%) | 0.032 |
| | age ≥48 | 56 (44.8%) | 33 (26.4%) | 23 (18.4%) | | 42 (33.6%) | 14 (11.2%) | |
| gender | male | 34 (27.2%) | 17 (13.6%) | 17 (13.6%) | 0.147 | 26 (20.8%) | 8 (6.4%) | 0.282 |
| | female | 91 (72.8%) | 60 (48%) | 31 (24.8%) | | 78 (62.4%) | 13 (10.4%) | |
| pT | pT 1-2 | 55 (44%) | 40 (32%) | 15 (12%) | 0.027 | 51 (40.8%) | 4 (3.2%) | 0.015 |
| | pT 3-4 | 70 (56%) | 37 (29.6%) | 33 (26.4%) | | 53 (42.4%) | 17 (13.6%) | |
| LN | no metastasis | 104 (83.2%) | 74 (59.2%) | 30 (24%) | 0.0001 | 90 (72%) | 14 (11.2%) | 0.049 |
| | metastasis | 21 (16.8%) | 3 (2.4%) | 18(14.4%) | | 14 (11.2%) | 7 (5.6%) | |
| OS | censored | 111 (88.8%) | 75 (60%) | 36 (28.8%) | 0.0001 | 97 (77.6%) | 14 (11.2%) | 0.002 |
| OS | death | 14 (11.2%) | 2 (1.6%) | 12 (9.6%) | | 7 (5.6%) | 7 (5.6%) | |
| TSS | censored | 118 (94.4%) | 77 (61.6%) | 41 (32.8%) | 0.001 | 102 (81.6%) | 16 (12.8%) | 0.001 |
| TSS | death | 7 (5.6%) | 0 (0%) | 7 (5.6%) | | 2 (1.6%) | 5 (4%) | |
| RFS | censored | 68 (54.4%) | 56 (44.8%) | 12 (9.6%) | 0.0001 | 66 (52.8%) | 2 (1.6%) | 0.0001 |
| RFS | relapse | 57 (45.6%) | 21 (16.8%) | 36 (28.8%) | | 38 (30.4%) | 19 (15.2%) | |

Table 3: Tall cells, BRAF and TERT

| | | total (n) | TC <10% (n) | TC ≥10% (n) | p-value | TC <50% (n) | TC ≥50% (n) | p-value |
|---|---|---|---|---|---|---|---|---|
| BRAF pyro | wild type | 46 (39%) | 37 (31.4%) | 9 (7.6%) | 0.001 | 41 (34.7%) | 5 (4.2%) | 0.143 |
| | mutated | 72 (61%) | 35 (29.7%) | 37 (31.4%) | | 56 (47.5%) | 16 (13.6%) | |
| BRAF antibody | wild type | 62 (49.6%) | 45 (36%) | 17 (13.6%) | 0.017 | 53 (42.4%) | 9 (7.2%) | 0.633 |
| | mutated | 63 (50.4%) | 32 (25.6%) | 31 (24.8%) | | 51 (40.8%) | 12 (9.6%) | |
| BRAF total | wild type | 49 (39.2%) | 39 (31.2%) | 10 (8%) | 0.001 | 44 (35.2%) | 5 (4%) | 0.088 |
| | mutated | 76 (60.8%) | 38 (30.4%) | 38 (30.4%) | | 60 (48%) | 16 (12.8%) | |
| TERT | wild type | 102 (92.7%) | 65 (59.1%) | 37 (33.6%) | 0.004 | 89 (80.9%) | 13 (11.8%) | 0.0003 |
| | mutated | 8 (7.3%) | 1 (0.9%) | 7 (6.4%) | | 2 (1.8%) | 6 (5.5%) | |

Table and Figure Legends

Figure 1: A: Papillary cancer; tall cell variant: tumor cells are at least thrice as height as their width and show an eosinophilic cytoplasm and elongated follicles (HE, 200x)

B: Same tumor as A: Papillary cancer; tall cell variant: three times as height as width cells arranged in a “tram track” pattern with eosinophilic cytoplasm (HE, 400x)

C: Papillary cancer; tall cell variant: papillary growth pattern with delicate branching of papillae (HE, 200x)

D: Same tumor as C: Papillary cancer; tall cell variant: three times as height as width cells, growing in a classic papillary fashion (HE, 400x)

E: Not all cells in these tumors are at least three times high as width. However, tumor cells growing in the “tram track” pattern should trigger a careful search for tall cells (HE, 200x)

F: Same tumor as E: Tumor cells are cut tangential to their base (upper). Only cells can be assessed where the basal membrane is seen (lower). These tumor cells are classic tall cells, often with nuclear grooves and elongated follicles (HE, 400x)

Figure 2: A: Kaplan Meier, overall survival; tall cells <10% and >=10%; Log Rank p<0.001; TC <10%: Mean ± Std. error: 200.07 ± 7.37; 95%CI: 185.62 – 214.52; TC >=10%: Mean ± Std. error: 152.14 ± 14.90; 95%CI: 122.93 – 181.34.

B: Kaplan Meier, relapse free survival; tall cells <10% and >=10%; Log Rank p<0.001; TC <10%: Mean ± Std. error: 150.98 ± 10.9; 95%CI: 129.63 – 172.32; TC >=10%: Mean ± Std. error: 40.20 ± 7.0; 95%CI: 26.49 – 53.91.

C: Kaplan Meier, overall survival; tall cells <10% vs. >=10% - <50%, Log Rank p<0.001 and tall cells >=10% - <50% vs. >=50%; Log Rank p<0.518;

TC <10%: Mean ± Std. error: 200.07 ± 7.37; 95%CI: 185.62 – 214.52; TC >=10% - <50%: Mean ± Std. error: 162.48 ± 14.94; 95%CI: 133.20 – 191.76; TC >=50%: Mean ± Std. error: 138.85 ± 20.18; 95%CI: 99.30 – 178.40.

D: Kaplan Meier, relapse free survival; tall cells <10% vs. >=10% - <50%, Log Rank p<0.001; and tall cells >=10% - <50% vs. >=50%; Log Rank p<0.379;

TC <10%: Mean ± Std. error: 150.98 ± 10.9; 95%CI: 129.63 – 172.32; TC >=10% - <50%: Mean ± Std. error: 50.32 ± 10.87; 95%CI: 29.01 – 71.63; TC >=50%: Mean ± Std. error: 32.43 ± 8.58; 95%CI: 15.62 – 49.24.

Figure 3: The influence of *BRAF* (A – C) and *TERT* promoter mutations (D – F) on overall survival, tumor specific survival or relapse free survival in patients with PTC; all Log Rank test

A; Kaplan Meier, overall survival, *BRAF* wt Mean ± Std. error: 191.75 ± 11.04; 95%CI: 170.11 – 213.38; *BRAF V600E*: Mean ± Std. error: 175.60 ± 10.30; 95%CI: 155.40 – 195.79

B; Kaplan Meier, tumor specific survival, *BRAF* wt Mean ± Std. error: 210.22 ± 4.65; 95%CI: 201.10 – 219.33; *BRAF V600E*: Mean ± Std. error: 187.34 ± 9.30; 95%CI: 169.11 – 205.58

C; Kaplan Meier, relapse free survival, *BRAF* wt Mean ± Std. error: 121.822 ± 12.76; 95%CI: 96.82 – 146.82; *BRAF V600E*: Mean ± Std. error: 96.99 ± 12.08; 95%CI: 73.31 – 120.67

D; Kaplan Meier, overall survival, *TERT* promoter wt Mean ± Std. error: 196.64 ± 8.84; 95%CI: 169.31 – 203.96; *TERT* promoter mutated: Mean ± Std. error: 107.18 ± 19.80; 95%CI: 68.37 – 145.99

E; Kaplan Meier, tumor specific survival, *TERT* promoter wt Mean ± Std. error: 200.60 ± 6.39; 95%CI: 188.08 – 213.11; *TERT* promoter mutated: Mean ± Std. error: 127.63 ± 13.38; 95%CI: 101.40 – 153.86

F; Kaplan Meier, relapse free survival, *TERT* promoter wt Mean ± Std. error: 101.34 ± 9.09; 95%CI: 83.52 – 119.16; *TERT* promoter mutated: Mean ± Std. error: 16.13 ± 6.15; 95%CI: 4.07 – 28.18

Figure suppl.1: ROC curves for tall cells in the different tumors. Results for the different survival endpoints are all significant.

Table 1: Clinical and pathological characteristics between the adverse clinical outcome group (ACO) and the control group (CG); (Chi-Square test).

Table 2: Clinicopathological characteristics of the study population for PTC (Papillary thyroid carcinoma) with different cutoffs for the TCV (Tall cell variant of papillary thyroid carcinoma); TC = Tall cells, OS = overall survival, TSS = tumor specific survival, RFS = relapse free survival, (Chi-Square test).

Table 3: Tall cells, *BRAF* and *TERT* promoter mutation status of the study population for PTC (Papillary thyroid carcinoma) with different cutoffs for the TCV.
BRAF pyro = *BRAF V600E* mutational status as detected by pyrosequencing; BRAF antibody = *BRAF V600E* mutational status as detected by the mutation specific VE1 antibody for *BRAF V600E*; BRAF total = *BRAF V600E* mutational status as detected by pyrosequencing when available and otherwise by the mutation specific VE1 antibody for *BRAF V600E*; (Chi-Square test).

Table suppl.1: Percentage of TC areas in different tumors and number of patients with different survival events.

Table suppl.2: Correlations and significances between different important clinicopathological data (Kendall's tau_b). **Correlation is significant at the 0.01 level (2-tailed); *Correlation is significant at the 0.05 level (2-tailed).

Table suppl.3: Detailed results of different multivariate analyses (Cox regression).